N, N-Dimethylacetamide, an FDA approved excipient, acts post-meiotically to impair spermatogenesis and cause infertility in rats

Khera, Nupur ; Ghayor, Chafik ; Lindholm, Anna K ; Pavlova, Ekaterina ; Atanassova, Nina ; Weber, Franz E

Abstract: N, N-Dimethylacetamide is an FDA approved solvent widely used in pharmaceutical industry to facilitate the solubility of lipophilic, high molecular weight drugs with poor water solubility. However, the cytotoxic effects of DMA raises the concern about its use in clinical applications. In the present study, we address the effect of DMA on spermatogenesis. Male Sprague Dawley rats were injected intra-peritoneally for 8 weeks, once a week at a dose of 862 mg/kg. Analysis of reproductive parameters revealed that DMA treated animals exhibit spermatid formation defects within the testis describing the characteristics of oligozoospermia. A subsequent decrease in epididymal sperm concentration along with distortion of sperm morphology was observed. The mitochondrial and microtubule organization in the sperm is considerably modified by DMA. This disrupts the sperm kinetics thus decreasing the total and progressive sperm motility. Finally, DMA treatment resulted in loss of fertility. Our results indicate that exposure to DMA has a negative impact on spermatogenesis and leads to infertility in male rats by inhibiting the post meiotic stages of sperm development. Therefore, the use of DMA in humans must be closely monitored.

DOI: https://doi.org/10.1016/j.chemosphere.2020.127001

Chemosphere 256 (2020) 127001

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

N, N-Dimethylacetamide, an FDA approved excipient, acts post-meiotically to impair spermatogenesis and cause infertility in rats

Nupur Khera [a, b], Chafik Ghayor [a], Anna K. Lindholm [c], Ekaterina Pavlova [d], Nina Atanassova [d], Franz E. Weber [a, b, e, *]

[a] University of Zurich, Center of Dental Medicine, Oral Biotechnology & Bioengineering, Plattenstrasse11, 8032, Zürich, Switzerland
[b] Zurich Center for Integrative Human Physiology (ZIHP), University of Zurich, Switzerland
[c] Department of Evolutionary Biology and Environmental Studies, University of Zurich, Zurich, Switzerland
[d] Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia, Bulgaria
[e] CABMM, Center for Applied Biotechnology and Molecular Medicine, University of Zurich, Zürich, Switzerland

HIGHLIGHTS

- N, N-Dimethylacetamide (DMA) alters the ultrastructure of acrosome leading to infertility in rats.
- DMA impairs spermatogenesis by inhibiting the post-meiotic round and elongated spermatids within the testis.
- DMA distorts the mitochondrial and microtubular structure of sperm therefore, reducing the sperm motility.

ARTICLE INFO

Article history:
Received 11 March 2020
Received in revised form
5 May 2020
Accepted 5 May 2020
Available online 15 May 2020

Handling Editor: A. Gies

Keywords:
N
N-Dimethylacetamide
Reproductive toxicology
Male infertility

ABSTRACT

N, N-Dimethylacetamide is an FDA approved solvent widely used in pharmaceutical industry to facilitate the solubility of lipophilic, high molecular weight drugs with poor water solubility. However, the cytotoxic effects of DMA raises the concern about its use in clinical applications. In the present study, we address the effect of DMA on spermatogenesis. Male Sprague Dawley rats were injected intraperitoneally for 8 weeks, once a week at a dose of 862 mg/kg. Analysis of reproductive parameters revealed that DMA treated animals exhibit spermatid formation defects within the testis describing the characteristics of oligozoospermia. A subsequent decrease in epididymal sperm concentration along with distortion of sperm morphology was observed. The mitochondrial and microtubule organization in the sperm is considerably modified by DMA. This disrupts the sperm kinetics thus decreasing the total and progressive sperm motility. Finally, DMA treatment resulted in loss of fertility. Our results indicate that exposure to DMA has a negative impact on spermatogenesis and leads to infertility in male rats by inhibiting the post meiotic stages of sperm development. Therefore, the use of DMA in humans must be closely monitored.

1. Introduction

N, N-Dimethylacetamide (DMA) is a widely used solvent due to its relatively high boiling point, thermal stability and high purity (Solomon et al., 1991). In industrial setting, it is applied for the manufacturing of films and fibers (Malley et al., 1995). However, exposure to DMA must be controlled due to its cytotoxic effects. The primary organ following high-level exposure is the liver, where the degree of damage is proportional to the exposure levels. Exposure to massive doses can lead to damage to other organs as well (Kennedy, 1986). In addition to the chemical industry, DMA is a widely used solvent in the pharmaceutical industry. It is the choice of solvent in the manufacturing process of the antibiotic cefpodoxime proxetil, administered for the treatment of respiratory and urinary tract infections (Rodriguez et al., 2003). DMA facilitates at a high dose the preparation of intravenous formulations of busulfan, a chemotherapeutic agent administered prior to hematopoietic stem cell transplantation or bone marrow transplantation in both adults and children (Hassan, 1999; Trame et al., 2013). Ideally, as a

* Corresponding author. University Zurich, Center of Dental Medicine/MKG, Oral Biotechnology & Bioengineering, Plattenstrasse 11, 8032, Zürich, Sweden.
E-mail address: franz.weber@zzm.uzh.ch (F.E. Weber).

https://doi.org/10.1016/j.chemosphere.2020.127001

pharmaceutical excipient, DMA should be devoid of any biological activity. However, in 2017, we showed that DMA binds bromodomains and may be useful for the treatment of osteoporosis (Ghayor et al., 2017). Subsequently, we have shown that it can act as a therapy for TNF-α compromised bone healing (Chen et al., 2019). Further research has also focused on its role in female reproduction where it regulates pro-inflammatory response associated with endotoxin to prevent preterm birth (Gorasiya et al., 2018). Together, these results show that DMA, being an organic solvent is biologically active. It acts via binding to bromodomain proteins. Bromodomain containing proteins are *trans*-acting readers that recognize acetylated lysine. They act as scaffolds to recruit macromolecular complexes that alter the chromatin accessibility to transcriptional machinery (Prinjha et al., 2012). The BET (bromodomain and extra terminal) family of bromodomain containing proteins consist of an N-terminal bromodomain and a C-terminal ET (extra-terminal) domain that is unique to this group of proteins. The four mammalian BET proteins include BRD2, BRD3, BRD4 and the testis specific BET protein, BRDT (Florence and Faller, 2001). They are involved in various cellular processes including proliferation and differentiation. During spermatogenesis, each of the BET genes is expressed at specific time and are essential for proper spermatogenesis process (Taniguchi, 2016).

Spermatogenesis is a complex and tightly regulated process by which spermatozoa are produced from spermatogonial stem cells. This process involves mitosis of spermatogonia and meiosis of spermatocytes, leading to the production of spermatids. During spermiogenesis, the spermatids undergo significant morphological transformations, leading to the production of fully differentiated mature spermatozoa (Holstein et al., 2003). The progression through each of the stages is a co-ordinated action of multiple genes and proteins including the proteins from the BET family (Berkovits and Wolgemuth, 2013). BRD2 associates with transcription factor E2F to induce transcriptional activation of genes, especially during the late spermatocyte stages of spermatogenesis (Sinha et al., 2005). BRD4 plays an important role during spermiogenesis by forming a ring like structure around the spermatid nuclei (Bryant et al., 2015). Binding interference studies have shown that DMA reduced the binding capacity of BRD2 and BRD4 to the acetyl lysine sites (Ghayor et al., 2017). Previously, it has been shown that exposure to DMA results in a decrease in relative testis weight along with degeneration of seminiferous tubules (R. Valentine et al., 1997). However, the mechanism by which DMA affects spermatogenesis is still elusive.

In the present study, we show that intraperitoneal injections of DMA in rats cause morphological and transcriptional alterations in sperm by inhibiting the post meiotic stages of spermatogenesis. This raises a concern about DMA as an excipient in pharmaceutical formulations and its effect on the patient's reproductive health.

2. Methods

Animal experiments: All animal procedures met the ARRIVE guidelines approved by the Animal Ethics Committee of the local authorities (Veterinäramt, Canton Zurich, project codes: 40/2012 (approved on the 2 April 2012) and 068/2015 (approved on the 31 July 2015), and follow the EU Directive 2010/63/EU for animal. Male Sprague-Dawley rats (6 weeks old) were purchased from Charles River Laboratories. After acclimatizing the animals to the animal facility environment for two weeks, they were randomly divided into two groups: DMA treatment group and a control group (PBS). In three independent experiments, 16 animals (8 per group) were used for body, testis and epididymal weight and Computer Assisted Sperm Analysis. For subsequent experiments including histological analysis, TUNEL assay, qPCR analysis, ANV determination (germ cell counting), 5 animals from the initial eight animals per group were randomly chosen. Volume of DMA to be injected was one third of the LD_{50} (862 mg/kg). This dose was chosen from our previous studies of DMA on rats (Ghayor et al., 2017). In addition, a pilot study with $n = 3$ showed a significant decrease in sperm count and alteration of seminiferous epithelium (data not shown), therefore the chosen dose was continued. One third of the lethal dose was calculated as previously described (Bartsch et al., 1976). Animals were injected once per week, intra-peritoneally for eight consecutive weeks, which is equivalent to one spermatogenesis cycle in rats. The injection volume of each dose was 800 μl and animals were weighed weekly to calculate the actual volume of chemicals. The control animals received the same volume of PBS. Animals were checked three times a week for any physiological side effects. After eight weeks, the animals were sacrificed by CO_2 asphyxiation and the blood was collected by aortal puncture. The clotted blood was centrifuged at 2000 rpm for 20 min to collect the serum. The serum was stored at −80 for hormonal analysis. Liver, testis and epididymis were collected in appropriate medium for further analysis.

For mating experiments, one male was mated with one female and the number of pups born were recorded.

Hormonal analysis: Serum Follicular Stimulating Hormone (FSH), Luteinizing Hormone (LH) and Testosterone were measured using the immunoassay kits from Cloud Clone Corp. (Product No. CEA830Ra, CEA441Ra, CEA458Ge) according to manufacturer's protocol.

Computer Assisted Sperm Analysis (CASA): The left epididymis was collected in HTF medium (Irvine Scientific, Article number 90126). For sperm counting, caudal epididymis was carefully cut, punctured using a syringe needle and incubated in pre warmed HTF medium for 10 min at 37 °C. The sperm were allowed to swim out in 1 mL of HTF medium and then collected in a 1.5 mL Eppendorf tube. The 1 mL sperm sample was carefully mixed and was further diluted 1:100 (1 μL sperm sample and 99 μL HTF medium). 30 μL of the diluted sample was placed on a slide (Leja slides for motility and concentration analysis, Microptic) and observed under the microscope attached to Computer Assisted Sperm Analyser (Hamilton) that provided measure of the sperm count, motility, progressive motility and other parameters. All sperm parameters were measured in 10 different fields on the slide.

12 different CASA parameters were recorded. One measure of sperm number (sperm count) and two measures of sperm motility (percentage motility and progressive motility) were recorded. Percentage motility records the percentage of total progressive and non-progressively motile sperms. Progressive motility records the number of sperms that move in a straight line or large circles. Straight line velocity (VSL) records the time-average velocity of a sperm head along the straight line between its first and last detected positions. Curvilinear velocity (VCL) records the average velocity measured over the actual point-to-point track followed by the cell. The time-average velocity (VAP) records the sperm head along its spatial average trajectory (i.e. smoothed version of VCL). Amplitude of lateral head displacement (ALH) is the maximum lateral displacement of a sperm head about its spatial average trajectory (i.e. track width). Linearity (LIN) is the linearity of the curvilinear trajectory calculated as VSL/VCL × 100. Straightness (STR) is the LIN of the sperm average path calculated as VSL/VAP × 100. Beat cross frequency (BCF) is the time-average rate at which the curvilinear sperm track crosses its average path trajectory. The elongation ratio of minor to major axis of each sperm nucleus and area of sperm head in micrometre square were also recorded (Sloter et al., 2006).

Morphological analysis of sperm: For morphological analysis,

sperm were isolated from caudal epididymis as explained before. 10 μL of 1:100 diluted sperm suspension was smeared on a glass slide. The slides were dipped in bouin's solution (Sigma-Aldrich) overnight and then stained with 2% eosin solution (Sigma-Aldrich) for 5 min. The slides were observed under the microscope (Leica Microsystems, Wetzlar, Germany) for abnormalities. 200 sperms were analysed per sample in a blinded manner for abnormal shape of the head (broken head, pinhole head and flattened head), bent neck and tail abnormalities (headless tail, short tail) as previously described (Demirci and Sahin, 2019). Percentage of normal and abnormal sperms was recorded ((number of normal sperms or sperms having the abnormality/200) *100).

Transmission Electron Microscopy: Sperm isolated from cauda epididymis were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.35) for 1 h and rinsed three times with 0.1 M sodium cacodylate buffer for 5 min each. After centrifugation, the specimens were sequentially treated with 1% OsO_4 in 0.1 M sodium cacodylate buffer for 1 h at 0 °C and with 2% uranyl acetate in H_2O for 1 h at 4 °C, with three H_2O rinsing steps (5 min each) between each step. The final pellet was immobilized with 2% of Difco Noble Agar (BD Biosciences, Allschwil, Switzerland) in H_2O, dehydrated in an ethanol series and embedded in Epon/Araldite (Sigma-Aldrich, Buchs, Switzerland). Ultrathin (70 nm) sections were contrasted with Reynolds lead citrate and examined with a CM100 transmission electron microscope (Thermo Fisher Scientific, Eindhoven, The Netherlands) at an acceleration voltage of 80 kV using an Orius 1000 digital camera (Gatan, Munich, Germany).

Scanning Electron Microscopy: Sperm suspensions were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.35) and subsequently centrifuged onto 12 mm cover glasses using a Cytospin 2 centrifuge (Thermo Fisher Scientific, Schwerte, Germany) at 40 g for 6 min. Cover glasses with attached sperm were rinsed three times with PBS (pH 7.35) and post-fixed with 1% OsO_4 in PBS for 30 min and rinsed 2 times with PBS prior to dehydration in a sequence of increasing ethanol concentrations (70%, 100%). Samples were incubated in 100% hexamethylsilazane for 1 h and finally air dried overnight. Samples were mounted on aluminium stubs using conductive carbon tabs, coated with 4 nm of platinum in a CCU 010 sputtering device (Safematic, Bad Ragaz, Switzerland) and imaged in a Supra 50 VP scanning electron microscope at an acceleration voltage of 4 kV using the Everhart Thornley secondary electron detector (Zeiss, Oberkochen, Germany).

Haematoxylin and eosin staining: Testis and epididymis were collected in Bouin's solution. Liver was collected in formalin. The samples were sent to Sophistolab, Muttenz, Switzerland for preparation of histological sections and Haematoxylin and Eosin (H&E) stained slides. Briefly, the tissues were fixed in Bouin's solution, embedded in paraffin, sectioned using a microtome at 5 μm thickness. The sections were stained with Hematoxyllin and Eosin and were analysed blindly by 2 different experimenters.

Measurement of seminiferous tubule diameter: Twenty seminiferous tubules were randomly selected from each H&E stained testicular section, and the diameter was measured using the Cell Sense Entry™ software of CKX53 Olympus (Tokyo, Japan) microscope. The diameter was measured as the shortest distance between two parallel tangent lines of the outer edge of one tubule. The measurements were done blindly by the experimenter.

TUNEL Assay: Tunnel assay was performed to detect the apoptotic cells within the seminiferous tubules using the Dead-End™ Colorimetric TUNEL System (Promega USA) according to the manufacturer's protocol. TUNEL positive cells were counted in 20 random seminiferous tubules.

qPCR analysis: RNA was extracted using TRIzol reagent (Invitrogen). RNA quantity and quality ($A_{260}/_{A280}$ and A_{260}/A_{230}) was determined using Nanodrop 2000 Spectrophotometer (ThermoFisher). 1 μg of RNA per sample was reverse transcribed into cDNA using iScript reverse transcription supermix (Bio-Rad). RT-qPCR was performed using gene-specific primers (Supplementary Table) with SsoAdvanced universal SYBR green supermix and the CFX Connect real-time system (Bio-Rad). Gene expression was normalized to the reference gene beta actin using the comparative C_T method.

Mitochondrial Membrane Potential (MMP) measurement and Flow cytometry: Sperm suspension from caudal epididymis (10 million sperm/mL) was incubated with 100 nM of MitoTracker™ Deep Red FM (Invitrogen™) for 20 min at 37 °C in dark as previously described (Hallap et al., 2005). MitoTracker™ Deep Red FM was excited by 640 nm laser of BD FACS Canto II and the emission was recorded in 660/20 channel. Ten thousand events were recorded per sample. The decrease in MitoTracker™ Deep Red FM fluorescence in DMA compared to PBS was calculated for comparative analysis.

Cell counting for testis: For histological analyses, Bouin's fixed, paraffin embedded tissue sections were stained with H&E. Testicular cell composition was estimated using standard stereological techniques involving point counting of cell nuclei to determine the nuclear volume per testis of Sertoli cells and different maturational stages of germ cells, as previously described (Welsh et al., 2009). In brief, cross sections of testis from PBS control and DMA treated groups were examined using 63x objective fitted to Zeiss AxioScope A1 and a 121-point eyepiece graticule (Leica Microsystems, Wetzlar, Germany). Applying a systematic sampling pattern from a random starting point, 32 microscopic fields (3872 points) were counted for each animal. Points falling over Sertoli cell or germ cell nuclei, seminiferous epithelium, interstitium, seminiferous tubule lumen were scored, and they were expressed as relative (%) volume per testis. For Sertoli cells and germ cell types including spermatogonia (Sg), spermatocytes (Sc), round (rSd) and elongated (elSd) spermatids, the values for percent nuclear volume were converted to absolute nuclear volumes per testis by reference to testis volume (=weight) because shrinkage was minimal.

3. Results

DMA decreases testis weight and tubular diameter: After eight weeks of DMA treatment, the testis of DMA treated animals appeared slightly smaller than PBS controls (Fig. 1A). Although there was no change in the body weight, a significant decrease in testis weight was observed in DMA treated animals (1.80 ± 0.10 *vs* 1.61 ± 0.13) (Fig. 1B and C). Measurement of seminiferous tubule diameter and analysis of H&E sections of testis showed that the seminiferous tubular diameter was significantly lower in DMA treated animals compared to PBS. Magnified view of the seminiferous tubules revealed degeneration of germ cells especially in the inner layers of the tubule (Fig. 1D and E).

DMA affects spermatid formation within testis: To determine the germ cells that DMA targets, we used the standard stereological techniques to determine the ANV (Absolute Nuclear Volume) per testis for Spermatogonia (Sg), Spermatocytes (Sc), round spermatids (rSd), elongated spermatids (elSd), total spermatids (tSd) and Sertoli cells (SC). We found that while there was no significant change in ANV of Sg, Sc, rSd and SC; elSd in testis from DMA treated animals show a decrease of 31.14%. As a result, tSd (rSd and elSd) also decrease by 21.6% in DMA treated animals (Table 1).

Measurement of Apoptosis: To determine whether the decrease in testis weight, diameter of seminiferous tubules and degeneration of elongated spermatids in the testis can be linked to apoptosis, we performed TUNEL assay. A significant increase in number of tubules with apoptotic cells and number of TUNEL-positive cells per tubule after DMA treatment was observed

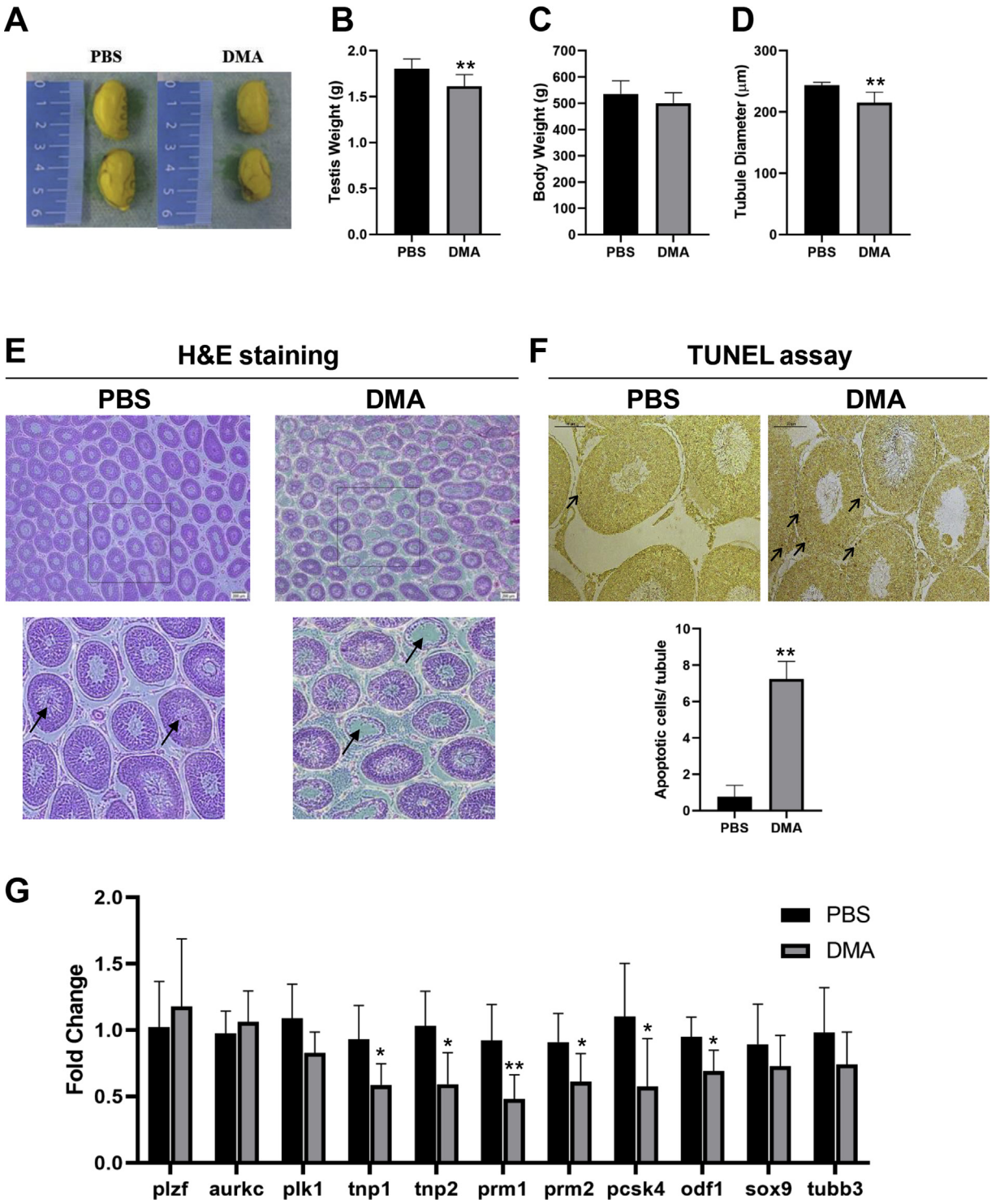

Fig. 1. Changes in testis weight, body weight, apoptosis and gene expression in PBS and DMA treated animals. (A) Representative image of gross anatomy of testis from PBS and DMA treated animals. (B) Effect of DMA treatment on testis weight (n = 8), (C) body weight (n = 8) and (D) Diameter of seminiferous tubules (n = 5). (E) H&E staining of testicular sections. Square indicates the enlarged image area. Black arrows indicate lumen of seminiferous tubules (scale bar: 200 μm) (n = 5). (F) Number of TUNEL positive cells per tubule as indicated by black arrows in PBS and DMA treated animals (Scale bar: 100 μm) (n = 5). (G) Quantitative RT-PCR analysis of genes expressed at different stages of sperm maturation and sertoli cells (n = 5). Data represents mean ± s.d. (*p < 0.05, **p < 0.01, obtained by two tailed student *t*-test). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(0.6 ± 0.55 *vs* 7.39 ± 1.00) (Fig. 1F).

Gene expression analysis: To study the changes at the level of gene expression, we performed qPCR for germ cell markers that play key roles at different stages of spermatogenesis: plzf for spermatogonia (Costoya et al., 2004), aurkc and plk1 for spermatocytes (Quartuccio and Schindler, 2015; Jordan et al., 2012), tnp1, tnp2, prm1, prm2, pcsk4 and odf1 for spermatids (Bao and Bedford, 2016; Gyamera-Acheampong and Mbikay, 2009; Yang

Table 1
Composition of different cell types in the testis from PBS and DMA treated animals.

Germ cell type		PBS	DMA
Total Germ cells	ANV (mg)	255.08 ± 25.34	237.154 ± 17.45
Spermatogonia	ANV (mg)	9.51 ± 3.30	9.35 ± 2.86
Spermatocytes	ANV (mg)	109.23 ± 14.90	113.47 ± 17.67
Total Spermatids	**ANV (mg)**	**139.56 ± 25.94**	**109.47 ± 27.38***
Round Spermatids	ANV (mg)	98.45 ± 25.99	74.36 ± 19.40
Elongated Spermatids	**ANV (mg)**	**47.19 ± 3.75**	**32.39 ± 8.20****
Sertoli cell	ANV (mg)	29.45 ± 6.39	29.05 ± 7.33

Data represent mean ± s.d. (*p < 0.05; **p < 0.01, obtained by two tailed student *t*-test), n = 5. ANV: Absolute Nuclear Volume.

et al., 2012), and sox9 and tubb3 for sertoli cells (Hemendinger et al., 2002; De Gendt et al., 2011). Although there was no change in gene expression of spermatogonia, spermatocytes and sertoli cells markers, a significant decrease in the expression of spermatids markers was observed in DMA treated animals (Fig. 1G).

DMA impairs sperm count and motility: Once elongated, the sperm produced within the testis, migrate to the epididymis to fully mature and gain motility (Bedford, 2015). To evaluate the effect of DMA on mature sperm leaving the testis, we analysed the epididymal parameters. After 8 weeks of injections, the weight of the epididymis was significantly reduced in DMA treated animals compared to PBS (1.01 ± 0.19 *vs* 0.74 ± 0.15) (Fig. 2A). Next, we performed CASA and observed a 73.6% decrease in sperm concentration in DMA treated animals compared to PBS (27.09 ± 20.33 million/mL *vs* 102.66 ± 32.46 million/mL) along with a 64.9% decrease in motility (Fig. 2B and C and supplementary videos 1 (PBS) and 2 (DMA)). The decrease in sperm count was consistent with the reduction in number of total and elongated spermatids within the testis. There was also a significant decrease in progressive motility (Fig. 2D). Moreover, we observed a significant reduction in the track speed (VCL) and area of sperm head (Table 2). Histological analysis of H&E stained epididymis sections confirmed the presence of a lower sperm count after DMA treatment (Fig. 2E).

Supplementary video related to this article can be found at https://doi.org/10.1016/j.chemosphere.2020.127001

DMA decreases Mitochondrial Membrane Potential (MMP): High sperm motility has been reported to be significantly associated with high mitochondrial functionality (Zhang et al., 2016). To test if the reduction in sperm motility observed in DMA treated group can be related to changes in mitochondrial membrane potential, we stained the sperm isolated from caudal epididymis with mitotracker deep red. Out of the total sperm, 29.22% of the DMA treated sperm showed lower mitotracker staining and therefore lower MMP compared to the PBS group (99.45 ± 0.15 *vs* 70.36 ± 5.10) (Fig. 3). This finding is consistence with the decrease in sperm motility after DMA treatment observed by CASA.

DMA causes abnormal morphogenesis of sperm: In addition to the quantitative loss of sperm, we observed morphological abnormalities in the sperm isolated from the cauda epididymis of DMA treated animals (Fig. 4A). This included abnormal shape of the

Table 2
Measurement of CASA parameters in epididymal sperm of PBS and DMA treated animals.

Parameter			PBS	DMA
Path Velocity	VAP	μm/s	195.5875 ± 16.86	164.275 ± 44.18
Prog. Velocity	VSL	μm/s	125.625 ± 8.20	113.875 ± 54.00
Track Speed	**VCL**	**μm/s**	**387.025 ± 37.63**	**301.0625 ± 59.87****
Lateral Amplitude	ALH	μm	18.7625 ± 1.59	15.8625 ± 5.09
Beat Frequency	BCF	Hz	29.0125 ± 1.40	28.3125 ± 4.64
Straightness	STR	%	61.75 ± 2.76	66.25 ± 7.53
Linearity	LIN	%	33.375 ± 2.13	39.25 ± 9.97
Elongation		%	28.625 ± 3.78	25.625 ± 6.94
Area		**μm sq**	**308.975 ± 67.97**	**162.375 ± 52.65****

Data represent mean ± s.d. (**p < 0.01, obtained by two tailed student *t*-test) (n = 8).

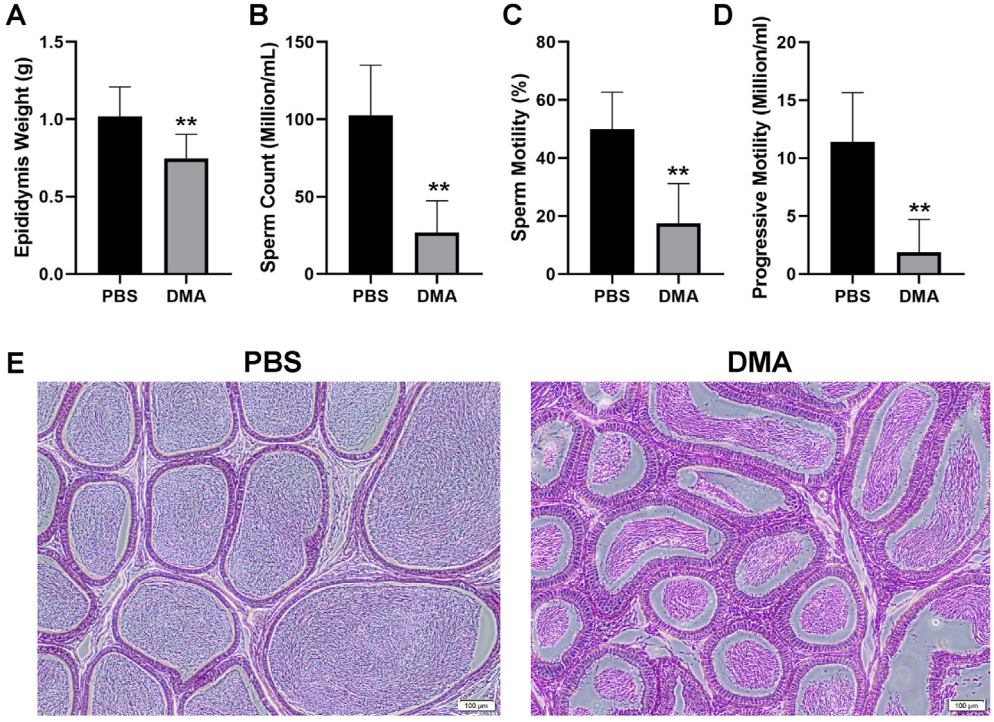

Fig. 2. Effect of DMA on sperm count and motility (A) Changes in epididymis weight (n = 8), sperm count, (C) sperm motility and (D) progressive motility in PBS and DMA treated animals (n = 8). (E) H&E staining of epididymis sections (scale bar: 100 μm) (n = 5). Data represents mean ± s.d. (**p < 0.01, obtained by two tailed student *t*-test). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

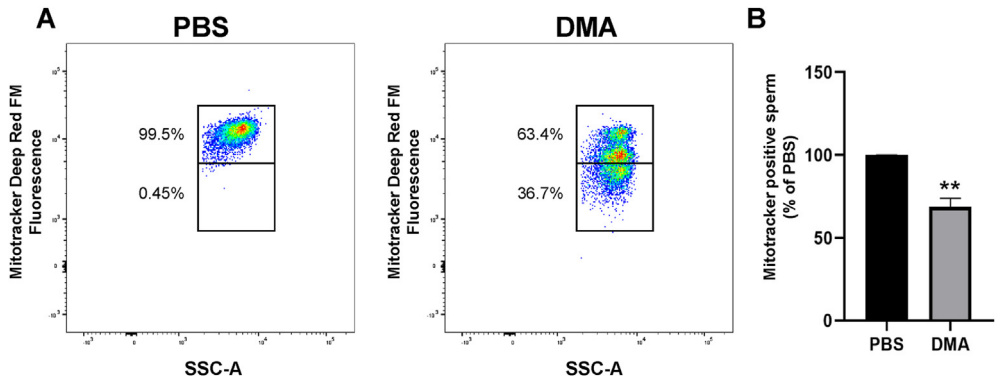

Fig. 3. DMA treatment affects mitochondrial membrane potential (MMP): (A) Representative images of Mitotracker deep red dot plots for PBS and DMA treated animals. (B) Number of Mitotracker positive sperm in DMA compared to PBS controls (n = 3). Data represents percentage of PBS. (**p < 0.01 obtained by two tailed student *t*-test). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

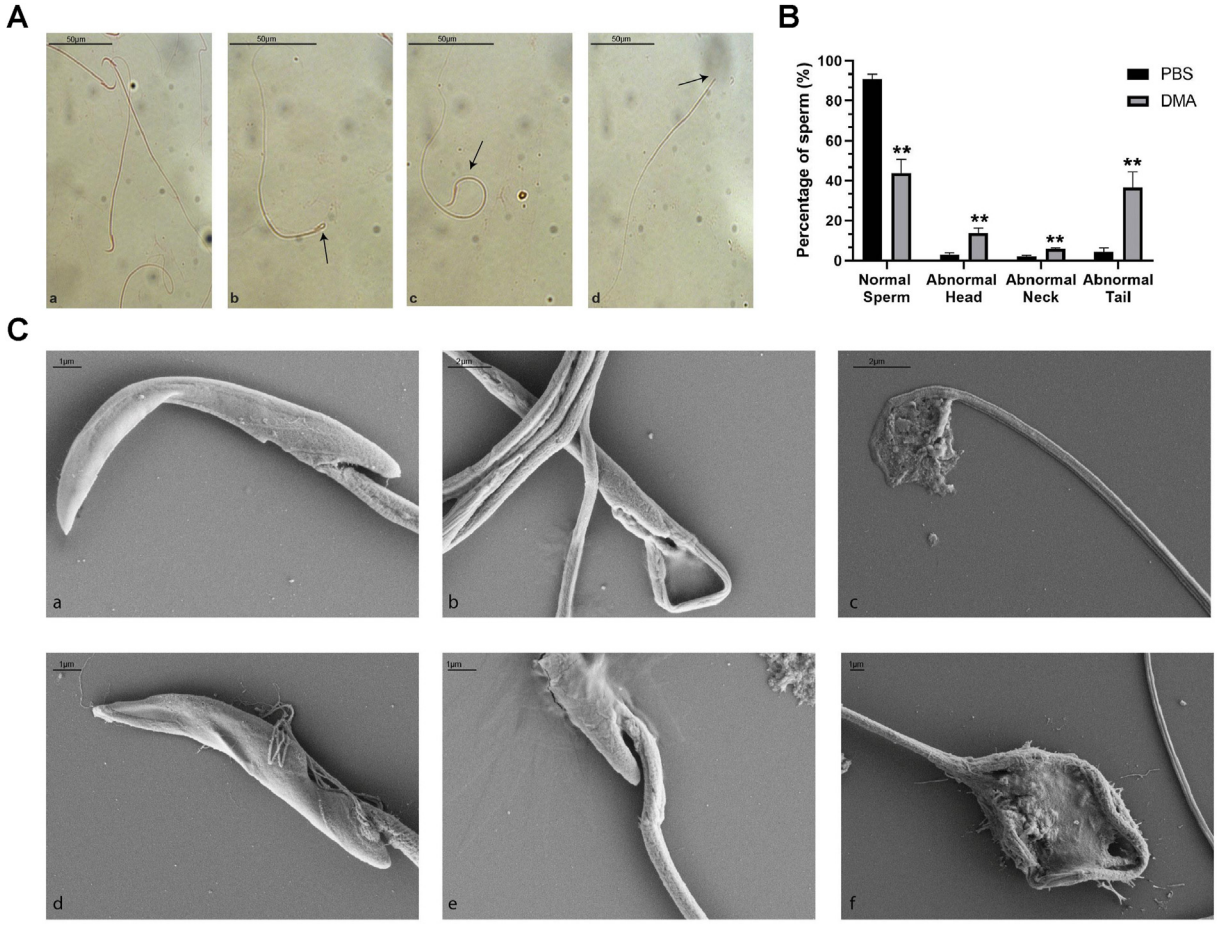

Fig. 4. Abnormal morphogenesis of sperm in DMA treated animals. (A) Morphology of (a) normal PBS and (b–d) abnormal DMA treated sperm under light microscope. Sperm were stained with eosin. Black arrows represent distorted head (b), neck (c) and (d) tail structures after DMA treatment (Scale bar: 50 μm). (B) Percentage of normal and abnormal sperm in PBS and DMA treated animals (n = 3). (C) SEM images of sperm heads from (a) PBS controls and (b–f) DMA treated animals (scale bar a,d,e,f: 1 μm; b,c: 2 μm). Data represents mean ± s.d. (**p < 0.01, obtained by two tailed student *t*-test). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

head (broken head, pinhole head and flattened head), bent neck and tail abnormalities (headless tail, short tail). Quantification results indicated that nearly 56% of the total sperm had at least one abnormality after DMA treatment (Fig. 4B). Scanning electron micrographs showed multiple malformations of the DMA treated head as well (Fig. 4C).

To determine the appearance of chromatin, the organization of microtubules and the acrosome structure, we performed ultrastructural analysis of sperm isolated from cauda epididymis by transmission electron microscopy. We observed appearance of multiple holes within the nucleus of sperm in DMA treated group. Compared to PBS group, DMA treatment compromised the

structure of middle piece and acrosome (Fig. 5). Epididymal sperm from PBS animals had normal "9 + 2" motile cilia axonemal structure. Other accessory structures were also well organised. However, in DMA treated animals, sperm had multiple axonemal anomalies like incomplete "9 + 2" core axonemal structure, mis-positioned outer dense fibers (ODF) and improper spatial organization of the middle piece and mitochondrial structure (Fig. 5). All these anomalies are associated with motility problems.

DMA causes infertility in rats: The head and acrosome structure are required for fertilization of the egg (Bhakta et al., 2019). Since these structures were compromised after DMA treatment, the effect of DMA was observed on fertility of these rats. Male rats were injected intraperitoneal with either PBS or DMA. After eight weeks, the injections were stopped, and each male was mated with one female. Both PBS and DMA produced equal number of litters. However, DMA treated animals produced a significantly lower number of pups (Fig. 6A). In the next set of experiments, additional injections for three more weeks after the initial 8 weeks resulted in complete infertility in DMA treated animals as no pups were born (Fig. 6B) despite proper mating as was evident by presence of

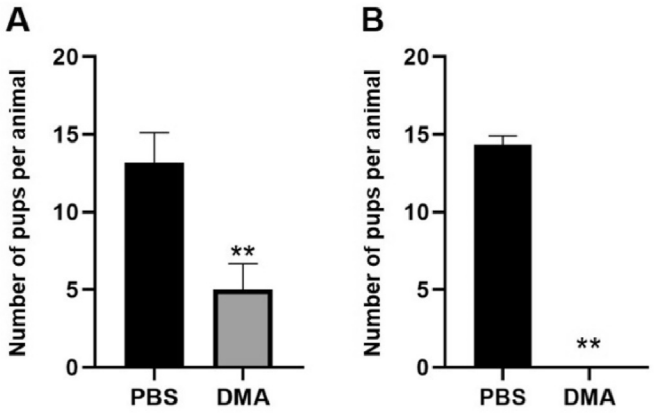

Fig. 6. Effect of DMA on fertility: Number of pups born per animal in PBS and DMA treated animals after (A) 8 weeks (n = 6) and (B) 11 weeks (n = 3) of treatment. Data represents mean ± s.d. (**p < 0.01, obtained by two tailed student *t*-test).

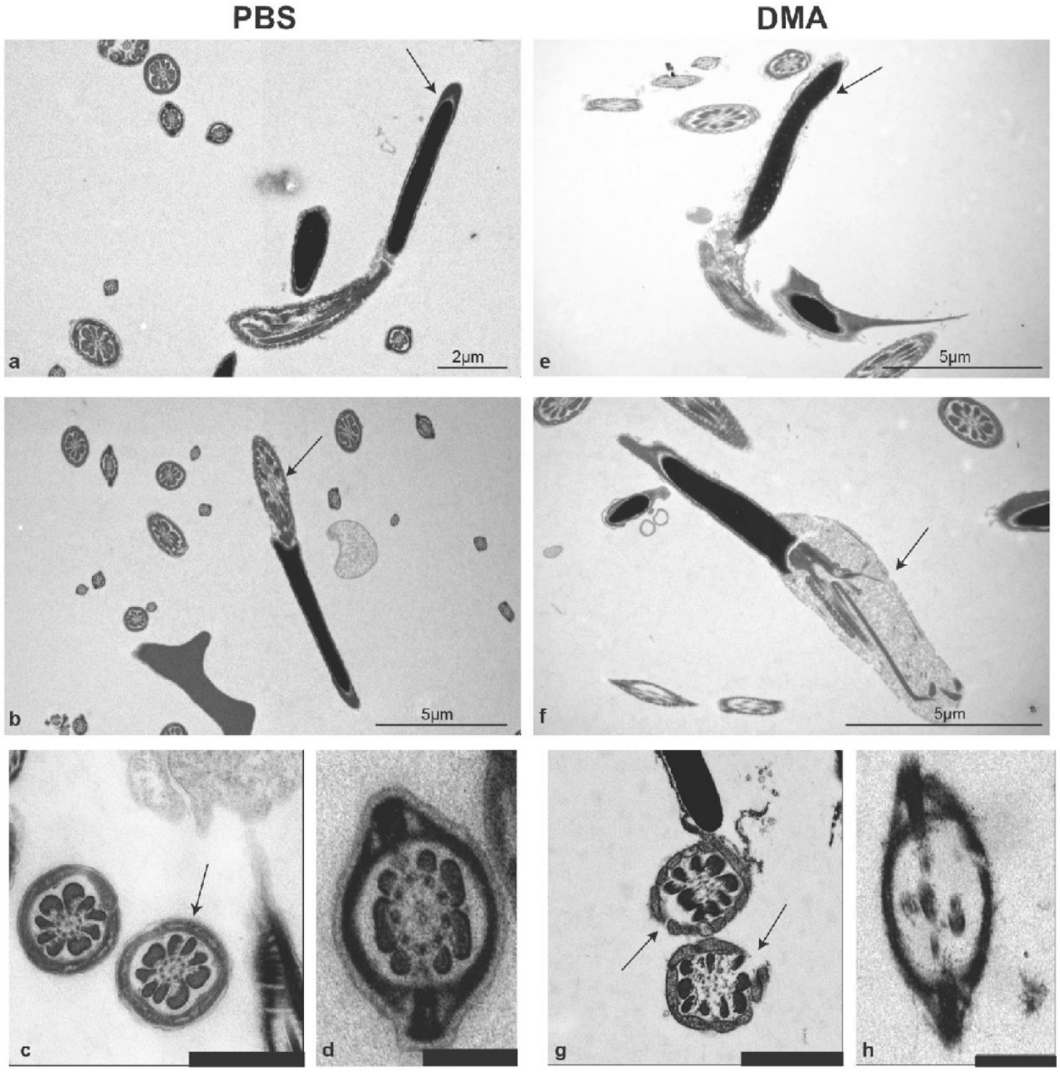

Fig. 5. Ultrastructural analysis of sperm using Transmission electron microscopy (TEM): PBS animals have (a) normal acrosomal layer around the nucleus, (b) properly organised middle piece, (c) outer dense fibers, mitochondria (black arrows) and (d) 9 + 2 microtubule structures. Sperm from DMA treated animals show (e) abnormal nucleus with multiple holes and degenerated acrosomal layer, (f) distorted middle piece, (g) dis-organised mitochondria and outer dense fibers, (Black arrows) and (h) abnormal 9 + 2 microtubule structure (n = 3). Scale bar: a: 1 μm; b,c and d: 5 μm; c and g: 1 μm; d and h: 300 nm.

sperms on vaginal smears before the 21 days gestation period. (Supplementary Fig. 1).

DMA does not alter the levels of sexual hormones: To determine if the loss of fertility after DMA treatment can be attributed to changes in sexual hormonal levels, we performed ELISA for Follicular stimulating hormone, Luteinizing Hormone and testosterone in serum. We found no significant change in serum levels of FSH, LH or testosterone (Fig. 7).

4. Discussion

In the current study, we have shown that intra-peritoneal injections of DMA affect spermatogenesis and cause infertility. Our results also indicate which phase of male germ cell development is disturbed by DMA treatment. Germ cell counting and histological analysis showed a decrease in number of total and elongated spermatids, and degeneration of inner layers of seminiferous tubules respectively. However, the outer layers were preserved without changing the number of spermatogonia or spermatocytes. Concomitantly, the gene expression analysis showed no change in expression of spermatogonia and spermatocyte markers but a significant decrease in post meiotically expressed genes in DMA treated animals. All these results concluded that DMA acts in a post meiotic manner to inhibit spermatogenesis.

A decrease in epididymal sperm concentration, deriving from a decrease in total and elongated spermatid counts within the testis displayed characteristic oligozoospermia (Guerri et al., 2019) in rats injected with DMA. Oligozoospermia is often followed by poor sperm motility and abnormal morphology reflecting qualitative and quantitative defects in spermatogenesis (McLachlan, 2013). Similar results were observed in sperm after DMA treatment.

Previous studies have shown that either genetic knockdown of testis specific BET protein, BRDT or its inhibition by small molecule JQ1 affects spermatogenesis. BRDT is expressed during pachytene spermatocyte, diplotene spermatocyte, and round spermatid stages (Shang et al., 2007; Matzuk et al., 2012). To examine the effect of DMA on BRDT we performed Alpha screen assay and observed that DMA inhibits BRDT with a half-maximal concentration (IC_{50}) of approximately 21 mM (Supplementary Fig. 2). Such a high IC_{50} value indicates that the effects caused by DMA are not specific to BRDT inhibition, since the peak-concentration of DMA in rats just after injection is 9.8 mM. This result was consistent with the germ cell counting where we observed that DMA has no effect on the number of spermatocytes or round spermatids within the seminiferous tubules, where BRDT is expressed.

While BRDT is expressed maximally in spermatocytes, the expression of BRD4 is increased during spermiogenesis. BRD4 forms a ring like structure around the acrosome. Acrosomal mutant mice lack this ring structure and show characteristic features of human globozoospermia leading to improper sperm elongation and infertility (Bryant et al., 2015). In a previous study, our lab has shown that DMA inhibits BRD4 with a half-maximal concentration (IC_{50}) of 6 mM (Ghayor et al., 2017). This explains the effects of DMA treatment on spermatogenesis including compromised acrosomal structure, decreased number of total and elongated spermatids, and loss of partial or complete fertility.

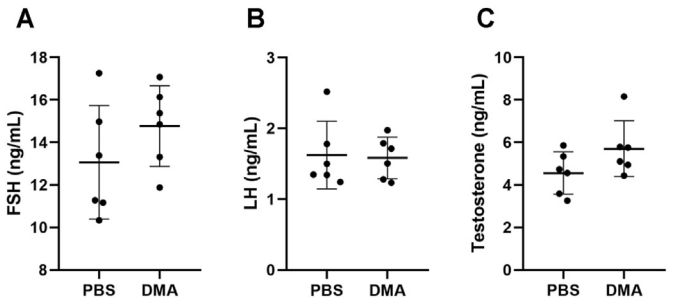

Fig. 7. Serological analysis of hormonal levels (A) Serum FSH, (B) Serum LH and (C) Serum testosterone levels in PBS and DMA treated animals (n = 6). Data represents mean ± s.d. Each individual dot represents one animal.

It was of interest to note that DMA treated animals share features with previously studied animal models that were deficient with genes expressed during post meiotic stages. A significant decrease in sperm motility and reduced fertility is observed in DMA treated animals as well as in tnp1 deficient mouse models (Yu et al., 2000). Multiple sperm head abnormalities and malformed acrosomal layer around the nucleus was common feature of both DMA treated animals and tnp2 null mice (Adham et al., 2001). Loss of ODF1, one of the main proteins of sperm tail results in formation of multiple headless tails. Morphological analysis of sperm from DMA treated animals showed similar tail abnormalities. In addition, misorganised mitochondrial sheath was also a shared characteristic for both odf1 deficient mice and DMA treated animals (Yang et al., 2012). We observed a significant decrease in gene expression of tnp1, tnp2 and odf1 in testis of DMA treated animals. This explains the similarities of compromised reproductive parameters in DMA treated and tnp1, tnp2 and odf1 deficient animals.

Previous studies have discussed about the hepatotoxic effects of DMA in rodents (Horn, 1961; Hundley et al., 1994; Solomon et al., 1991). Data on toxic effects of DMA in humans is scarce. In humans, DMA is injected as a solubilizer, along with busulfan treatment in children during chemotherapy. The total concentration of DMA administered per regimen in a patient can be up to 105 mmoL (Trame et al., 2013; Oechtering et al., 2006). Hempel et al. reported that DMA is not toxic even at high doses since it is rapidly cleared from the body. However, the effect of DMA on spermatogenesis was not monitored in that study (Hempel et al., 2007). By injecting DMA in rats, at a much lower dose than that used in humans, we have shown that this small chemical inhibits spermatogenesis.

5. Conclusion

In conclusion, the present study demonstrates that DMA, an FDA approved drug excipient, previously characterized as a bromodomain inhibitor, inhibits the spermatogenesis process leading to infertility in male rats. The observed oligozoospermia induced by DMA results from a decrease in number of total and elongated spermatids within the testis, thus acting only post-meiotically, leading to a decrease in sperm count in the epididymis. Furthermore, the sperm motility and mitochondrial membrane potential are also compromised. The consequence of the observed distortion in acrosomal structure is a partial or complete infertility of DMA treated animals. Since DMA is applied clinically at high dosages, the effect of DMA on spermatogenesis in humans during clinical applications should be closely monitored.

Authors' contributions

NK, CG and FEW designed the research; NK performed the research; NK and AL performed CASA experiments. EP and NA performed germ cell counting for testis. NK analysed the data. NK, CG, and FEW wrote the paper. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare no conflict of interests.

Acknowledgements

The authors thank Ana Perez, Flora Nicholls, Thea Fleischmann and Alexander Tchouboukov for the excellent technical assistance; Andres Kaech for scientific comments during microscopy experiments; and Tse-Hsiang Chen, Anja Ivica and Indranil Bhattacharya for their helpful advice. This project was supported by the Swiss Science Foundation with a grant (31003A_140868) to FEW.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.chemosphere.2020.127001.

References

Adham, I.M., Nayernia, K., Burkhardt-Gottges, E., Topaloglu, O., Dixkens, C., Holstein, A.F., Engel, W., 2001. Teratozoospermia in mice lacking the transition protein 2 (Tnp2). Mol. Hum. Reprod. 7, 513–520.

Bao, J., Bedford, M.T., 2016. Epigenetic regulation of the histone-to-protamine transition during spermiogenesis. Reproduction 151, R55–R70.

Bartsch, W., Sponer, G., Dietmann, K., Fuchs, G., 1976. Acute toxicity of various solvents in the mouse and rat. LD50 of ethanol, diethylacetamide, dimethylformamide, dimethylsulfoxide, glycerine, N-methylpyrrolidone, polyethylene glycol 400, 1,2-propanediol and Tween 20. Arzneimittelforschung 26, 1581–1583.

Bedford, J.M., 2015. The epididymis re-visited: a personal view. Asian J. Androl. 17, 693–698.

Berkovits, B.D., Wolgemuth, D.J., 2013. The role of the double bromodomain-containing BET genes during mammalian spermatogenesis. Curr. Top. Dev. Biol. 102, 293–326.

Bhakta, H.H., Refai, F.H., Avella, M.A., 2019. The molecular mechanisms mediating mammalian fertilization. Development 146.

Bryant, J.M., Donahue, G., Wang, X., Meyer-Ficca, M., Luense, L.J., Weller, A.H., Bartolomei, M.S., Blobel, G.A., Meyer, R.G., Garcia, B.A., Berger, S.L., 2015. Characterization of BRD4 during mammalian postmeiotic sperm development. Mol. Cell Biol. 35, 1433–1448.

Chen, T.H., Weber, F.E., Malina-Altzinger, J., Ghayor, C., 2019. Epigenetic drugs as new therapy for tumor necrosis factor-alpha-compromised bone healing. Bone 127, 49–58.

Costoya, J.A., Hobbs, R.M., Barna, M., Cattoretti, G., Manova, K., Sukhwani, M., Orwig, K.E., Wolgemuth, D.J., Pandolfi, P.P., 2004. Essential role of Plzf in maintenance of spermatogonial stem cells. Nat. Genet. 36, 653–659.

De Gendt, K., Denolet, E., Willems, A., Daniels, V.W., Clinckemalie, L., Denayer, S., Wilkinson, M.F., Claessens, F., Swinnen, J.V., Verhoeven, G., 2011. Expression of Tubb3, a beta-tubulin isotype, is regulated by androgens in mouse and rat Sertoli cells. Biol. Reprod. 85, 934–945.

Demirci, T., Sahin, E., 2019. The effect of chronic stress and obesity on sperm quality and testis histology in male rats; a morphometric and immunohistochemical study. Histol. Histopathol. 34, 287–302.

Florence, B., Faller, D.V., 2001. You bet-cha: a novel family of transcriptional regulators. Front. Biosci. 6, D1008–D1018.

Ghayor, C., Gjoksi, B., Dong, J., Siegenthaler, B., Caflisch, A., Weber, F.E., 2017. N,N Dimethylacetamide a drug excipient that acts as bromodomain ligand for osteoporosis treatment. Sci. Rep. 7, 42108.

Gorasiya, S., Mushi, J., Pekson, R., Yoganathan, S., Reznik, S.E., 2018. Repurposing N,N-dimethylacetamide (DMA), a pharmaceutical excipient, as a prototype novel anti-inflammatory agent for the prevention and/or treatment of preterm birth. Curr. Pharmaceut. Des. 24, 989–992.

Guerri, G., Maniscalchi, T., Barati, S., Busetto, G.M., Del Giudice, F., De Berardinis, E., Cannarella, R., Calogero, A.E., Bertelli, M., 2019. Non-syndromic monogenic male infertility. Acta Biomed. 90, 62–67.

Gyamera-Acheampong, C., Mbikay, M., 2009. Proprotein convertase subtilisin/kexin type 4 in mammalian fertility: a review. Hum. Reprod. Update 15, 237–247.

Hallap, T., Nagy, S., Jaakma, U., Johannisson, A., Rodriguez-Martinez, H., 2005. Mitochondrial activity of frozen-thawed spermatozoa assessed by MitoTracker Deep Red 633. Theriogenology 63, 2311–2322.

Hassan, M., 1999. The role of busulfan in bone marrow transplantation. Med. Oncol. 16, 166–176.

Hemendinger, R.A., Gores, P., Blacksten, L., Harley, V., Halberstadt, C., 2002. Identification of a specific Sertoli cell marker, Sox9, for use in transplantation. Cell Transplant. 11, 499–505.

Hempel, G., Oechtering, D., Lanvers-Kaminsky, C., Klingebiel, T., Vormoor, J., Gruhn, B., Boos, J., 2007. Cytotoxicity of dimethylacetamide and pharmacokinetics in children receiving intravenous busulfan. J. Clin. Oncol. 25, 1772–1778.

Holstein, A.F., Schulze, W., Davidoff, M., 2003. 'Understanding spermatogenesis is a prerequisite for treatment. Reprod. Biol. Endocrinol. 1, 107.

Horn, H.J., 1961. Toxicology of dimethylacetamide. Toxicol. Appl. Pharmacol. 3, 12–24.

Hundley, S.G., Lieder, P.H., Valentine, R., McCooey, K.T., Kennedy Jr., G.L., 1994. Dimethylacetamide pharmacokinetics following inhalation exposures to rats and mice. Toxicol. Lett. 73, 213–225.

Jordan, P.W., Karppinen, J., Handel, M.A., 2012. Polo-like kinase is required for synaptonemal complex disassembly and phosphorylation in mouse spermatocytes. J. Cell Sci. 125, 5061–5072.

Kennedy Jr., G.L., 1986. Biological effects of acetamide, formamide, and their monomethyl and dimethyl derivatives. Crit. Rev. Toxicol. 17, 129–182.

Malley, L.A., Slone Jr., T.W., Makovec, G.T., Elliott, G.S., Kennedy Jr., G.L., 1995. Chronic toxicity/oncogenicity of dimethylacetamide in rats and mice following inhalation exposure. Fund. Appl. Toxicol. 28, 80–93.

Matzuk, M.M., McKeown, M.R., Filippakopoulos, P., Li, Q., Ma, L., Agno, J.E., Lemieux, M.E., Picaud, S., Yu, R.N., Qi, J., Knapp, S., Bradner, J.E., 2012. Small-molecule inhibition of BRDT for male contraception. Cell 150, 673–684.

McLachlan, R.I., 2013. Approach to the patient with oligozoospermia. J. Clin. Endocrinol. Metab. 98, 873–880.

Oechtering, D., Boos, J., Hempel, G., 2006. Monitoring of N,N-dimethylacetamide in children during i.v.-busulfan therapy by liquid chromatography-mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 838, 129–134.

Prinjha, R.K., Witherington, J., Lee, K., 2012. Place your BETs: the therapeutic potential of bromodomains. Trends Pharmacol. Sci. 33, 146–153.

Quartuccio, S.M., Schindler, K., 2015. Functions of Aurora kinase C in meiosis and cancer. Front Cell Dev Biol 3, 50.

Rodriguez, J.C., Hernandez, R., Gonzalez, M., Rodriguez, Z., Tolon, B., Velez, H., Valdes, B., Lopez, M.A., Fini, A., 2003. An improved method for preparation of cefpodoxime proxetil. Farmaco 58, 363–369.

Shang, E., Nickerson, H.D., Wen, D., Wang, X., Wolgemuth, D.J., 2007. The first bromodomain of Brdt, a testis-specific member of the BET sub-family of double-bromodomain-containing proteins, is essential for male germ cell differentiation. Development 134, 3507–3515.

Sinha, A., Faller, D.V., Denis, G.V., 2005. Bromodomain analysis of Brd2-dependent transcriptional activation of cyclin A. Biochem. J. 387, 257–269.

Sloter, E., Schmid, T.E., Marchetti, F., Eskenazi, B., Nath, J., Wyrobek, A.J., 2006. Quantitative effects of male age on sperm motion. Hum. Reprod. 21, 2868–2875.

Solomon, H.M., Ferenz, R.L., Kennedy Jr., G.L., Staples, R.E., 1991. Developmental toxicity of dimethylacetamide by inhalation in the rat. Fund. Appl. Toxicol. 16, 414–422.

Taniguchi, Y., 2016. The bromodomain and extra-terminal domain (BET) family: functional anatomy of BET paralogous proteins. Int. J. Mol. Sci. 17.

Trame, M.N., Bartelink, I.H., Boos, J., Boelens, J.J., Hempel, G., 2013. Population pharmacokinetics of dimethylacetamide in children during standard and once-daily IV busulfan administration. Canc. Chemother. Pharmacol. 72, 1149–1155.

Valentine, R., Hurtt, M.E., Frame, S.R., Kennedy Jr., G.L., 1997. Inhalation toxicology of dimethylacetamide (DMAC) in mice and rats: age-related effects on lethality and testicular injury. Inhal. Toxicol. 9 (2), 41–156.

Welsh, M., Saunders, P.T., Atanassova, N., Sharpe, R.M., Smith, L.B., 2009. Androgen action via testicular peritubular myoid cells is essential for male fertility. Faseb. J. 23, 4218–4230.

Yang, K., Meinhardt, A., Zhang, B., Grzmil, P., Adham, I.M., Hoyer-Fender, S., 2012. The small heat shock protein ODF1/HSPB10 is essential for tight linkage of sperm head to tail and male fertility in mice. Mol. Cell Biol. 32, 216–225.

Yu, Y.E., Zhang, Y., Unni, E., Shirley, C.R., Deng, J.M., Russell, L.D., Weil, M.M., Behringer, R.R., Meistrich, M.L., 2000. Abnormal spermatogenesis and reduced fertility in transition nuclear protein 1-deficient mice. Proc. Natl. Acad. Sci. U. S. A. 97, 4683–4688.

Zhang, G., Wang, Z., Ling, X., Zou, P., Yang, H., Chen, Q., Zhou, N., Sun, L., Gao, J., Zhou, Z., Cao, J., Ao, L., 2016. Mitochondrial biomarkers reflect semen quality: results from the MARCHS study in chongqing, China'. PloS One 11, e0168823.